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Mutations in Pneumococcal *cpsE* Generated via *In Vitro* Serial Passaging Reveal a Potential Mechanism of Reduced Encapsulation Utilized by a Conjunctival Isolate

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ABSTRACT

The polysaccharide capsule of *Streptococcus pneumoniae* is required for nasopharyngeal colonization and for invasive disease in the lungs, blood, and meninges. In contrast, the vast majority of conjunctival isolates are acapsular. The first serotype-specific gene in the capsule operon, *cpsE*, encodes the initiating glycosyltransferase and is one of the few serotype-specific genes that can tolerate null mutations. This report characterizes a spontaneously arising TIGR4 mutant exhibiting a reduced capsule, caused by a 6-nucleotide duplication in *cpsE* which results in duplication of Ala and Ile at positions 45 and 46. This strain (AI45dup) possessed more exposed phosphorylcholine and was hypersusceptible to C3 complement deposition compared to the wild type. Accordingly, the mutant was significantly better at forming abiotic biofilms and binding epithelial cells *in vitro* but was avirulent in a sepsis model. *In vitro* serial passaging of the wild-type strain failed to reproduce the AI45dup mutation but instead led to a variety of mutants with reduced capsule harboring single nucleotide polymorphisms (SNPs) in *cpsE*. A single passage in the sepsis model after high-dose inoculation readily yielded revertants of AI45dup with restored wild-type capsule level, but the majority of SNP alleles of *cpsE* could not revert, suppress, or bypass. Analysis of *cpsE* in conjunctival isolates revealed a strain with a single missense mutation at amino acid position 377, which was responsible for reduced encapsulation. This study supports the hypothesis that spontaneous, nonreverting mutations in *cpsE* serve as a form of adaptive mutation by providing a selective advantage to *S. pneumoniae* in niches where expression of capsule is detrimental.

IMPORTANCE

While the capsule of *Streptococcus pneumoniae* is required for colonization and invasive disease, most conjunctival isolates are acapsular by virtue of deletion of the entire capsular operon. We show that spontaneous acapsular mutants isolated *in vitro* harbor mostly nonrevertible single nucleotide polymorphism (SNP) null mutations in *cpsE*, encoding the initiating glycosyltransferase. From a small collection of acapsular conjunctival isolates, we identified one strain with a complete capsular operon but containing a SNP in *cpsE* that we show is responsible for the acapsular phenotype. We propose that acapsular conjunctival isolates may arise initially from such nonreverting SNP null mutations in *cpsE*, which can be followed later by deletion of portions or all of the *cps* operon.

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium that is frequently found as a commensal organism of the human nasopharynx. However, depending on the site of dissemination, it can also cause various invasive diseases, including pneumonia, otitis media, sepsis, and meningitis (1–4). One critical pneumococcal virulence factor is the capsular polysaccharide (capsule), of which over 90 different serotypes have been characterized. The capsule has many important immune evasion functions, including avoidance of mucus-mediated clearance, entrapment in neutrophil extracellular traps (NETs), recognition by complement factors, and opsonophagocytosis (5–9). Although capsule is required for dissemination and invasive disease, during asymptomatic nasopharyngeal colonization, a reduced level of capsule is favored. Previous reports demonstrated that less encapsulated strains more effectively interact with host epithelial cells and form biofilms, both of which facilitate the persistence of pneumococcus in the nasopharynx (10–15). Interestingly, while capsule is necessary for invasive disease in the lungs, blood, and meninges, the vast majority of pneumococcal strains that cause conjunctivitis are acapsular (1–4, 16–18). These nontypeable (NT) conjunctival isolates are classified into two subgroups depending on the genetic structure of their capsule biosynthesis (*cps*) locus. Group I NT strains possess a *cps* locus very similar to that of encapsulated strains. Group II NT strains lack the vast majority of the *cps* locus and instead harbor other genes, such as *pspK* (encoding an LPXTG-anchored protein potentially involved in epithelial adhesion and invasion), *nspA* [encoding a novel surface protein which may interact with the human poly(Ig) receptor], and genes with significant sequence similarity to *aliB*, which encodes a pep-

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tide-binding molecule associated with an ABC transporter (19–23). While it is clear that the replacement of the entire *cps* locus results in an acapsular strain, the molecular mechanism(s) responsible for reduced/loss of capsule in conjunctival clinical isolates with a seemingly intact *cps* locus is incompletely understood.

The chemical composition and linkages between polysaccharide subunits distinguish one capsular serotype from another (10–15, 24–28). All serotypes, except for types 3 and 37, are synthesized in a Wzx flippase/Wzy polymerase-dependent fashion (1, 25, 29, 30). The *cps* locus consists of approximately 15 genes that encode all of the enzymes required for capsule synthesis and is arranged in a cassette structure positioned between the conserved genes *dexB* and *aliA*. Additionally, the entire *cps* operon is flanked by insertion sequences, which potentially facilitate the exchange of genetic material between pneumococcal strains, thus yielding capsular switch mutants (25, 31, 32). With the exception of serotypes 3 and 37, the first four genes, *cpsA* to *cpsD*, are highly conserved and encode regulatory proteins (25, 27, 33–35). The region downstream of *cpsD* is unique to each serotype and encodes glycosyl transferases, polymerases, transporters, enzymes involved in sugar nucleotide synthesis, Wzx flippase, and Wzy polymerase (27). Importantly, *cpsE* is the first serotype-specific gene and encodes the initiating glycosyltransferase that catalyzes the linkage of the sugar-phosphate to the lipid acceptor on the cytoplasmic face of the cell membrane (36–38). Mutations in *cpsE*, including simple sequence repeats within this gene, have been shown to reduce levels of encapsulation and may potentially represent another layer of complexity in the scheme of capsular polysaccharide regulation (36, 39–42).

This study begins with the characterization of a spontaneously arising mutant of TIGR4 (serotype 4) that exhibited reduced capsule expression. This mutant harbored a 6-bp duplication in *cps4E*, which was readily reversible following *in vivo* passage in the blood. Upon *in vitro* serial passaging of TIGR4 and screening for colony variants, additional unique mutants with reduced capsule expression were identified, all of which contained single nucleotide polymorphisms (SNPs) in *cps4E* representing missense or nonsense mutations. In contrast to the 6-bp duplication, the majority of the *cps4E* SNP mutations were not reversible following *in vivo* passage. From the available conjunctival isolates with an intact *cps* operon, we identified two serotype-matched (serotype 19A) variants containing SNP missense mutations in *cpsE*. One of these mutations was shown to be responsible for the acapsular phenotype by genetically reverting the mutation. This report describes the phenotypes for naturally occurring *cpsE* mutant populations and discusses the potential relevance to pneumococcal conjunctivitis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* TIGR4 and its unencapsulated derivative AC4421 were from our laboratory stocks. For indicated experiments, encapsulated and unencapsulated conjunctival isolates (serotype 19A) were utilized (43).

S. pneumoniae was grown in or on the following media at 37°C in a 5% CO_2 incubator: Todd-Hewitt yeast extract broth (THY), which was composed of Todd-Hewitt broth (Becton Dickinson, Co.) supplemented with 0.5% yeast extract (Fischer Scientific, Inc.) and 5 μl/ml Oxyrase (Oxyrase, Inc.); blood agar plates (BA), which are tryptic soy agar plates supplemented with 5% (vol/vol) sheep blood (Northeast Laboratories, Inc.); and Trypticase soy agar (TSA) plates supplemented with ~5,600 units of catalase (Worthington Biochemical Corporation). Where appropriate, streptomycin (Sm; 100 μg/ml) was added to the medium.

Serial passaging, screening of colonies, and sequencing of *cpsE* in capsule mutants. *S. pneumoniae* TIGR4 was plated on BA plates and grown overnight. Ninety-six randomly picked colonies were transferred using toothpicks to a 96-well, flat-bottom plate containing 100 μl of THY and incubated until the cultures reached mid-exponential phase, approximately 4 to 6 h. By using a 96-well pin replicator, the cultures were stamped onto a large BA plate and incubated overnight. This growth on BA plates and in THY was repeated for a total of 8 cycles. The final stamping of the 96 strains was done on TSA plates lacking blood to allow identification of bacterial growth with transparent morphology using oblique transmitted light microscopy.

Twenty-six stamped areas showing the transparent phenotype were restreaked for single colonies on TSA plates to visually confirm that the population was homogenously transparent. Unpassaged and passaged opaque colonies were also isolated as controls. Genomic DNA was isolated from these strains, and the *cps4E* gene was PCR amplified using primers in the flanking genes (Table 1). Sanger sequencing was used to determine the *cps4E* genotype of each strain of interest. To confirm that the *cps4E* mutation was solely responsible for the reduction in capsule level, the mutant allele was moved in a clean wild-type (WT) TIGR4 background, and the resultant panel of isogenic *cps4E* mutants was examined by India ink staining and microscopy. These *cps4E* mutants were further characterized using assays detailed below.

Construction of *cpsE* mutant strains. Mutant strains created during this study are described in Table 1 and were generated by allelic exchange. Each allelic exchange construct was generated *in vitro* using splicing by overlap extension PCR (44). The upstream and downstream arms of homology flanking *cpsE* were PCR amplified from TIGR4, MGMV 3105, or MGMV 3526 genomic DNA (gDNA) where appropriate. To aid in making *cpsE* mutants, a streptomycin resistance (Sm^r) construct, conferred by a point mutation in the *rpsL* gene (45), was also included during the transformation process. Transformation of *S. pneumoniae* was done as previously described (46). All *cpsE* mutations were confirmed by DNA sequencing, and mutants were confirmed to have reduced capsule levels by India ink staining.

C3 deposition, exposed phosphorylcholine (P-Cho), capsule assays, and fluorescence-activated cell sorting (FACS) analysis. For C3 deposition assays, 1 ml of mid-exponential-phase bacteria grown in THY was pelleted, washed in phosphate-buffered saline (PBS), and resuspended in 500 μl of Hanks buffer with Ca^{2+} and Mg^{2+} (Gibco Corp.) supplemented with 0.1% gelatin (Fischer Scientific, Inc.). A total of 10^7 CFU in 50 μl were added to a final concentration of 10% infant rabbit serum in 100 μl (AbD Serotec, Co.). Samples were incubated in a 37°C rolling incubator for 30 min. Next, opsonization reaction mixtures were chilled for 3 min on ice, quenched with 500 μl of Hanks buffer without Ca^{2+} and Mg^{2+} (Gibco) with 0.1% gelatin, and pelleted at 4,000 rpm for 5 min. Pellets were resuspended in 1:200 fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit C3 antibody (MP Biomedicals) in 100 μl Hanks buffer without Ca^{2+} and Mg^{2+} with 0.1% gelatin and incubated on ice in the dark for 30 min. Staining reactions were quenched with 500 μl Hanks buffer with 0.1% gelatin but without Ca^{2+} and Mg^{2+}, the mixtures were centrifuged at 4,000 rpm for 5 min, and pellets were resuspended in 300 μl of 2% paraformaldehyde (PFA; Sigma-Aldrich Co.).

For exposed P-Cho and capsule assays, 250 μl of mid-exponential-phase bacteria grown in THY were pelleted and washed in PBS. Pellets were resuspended in 100 μl of unconjugated mouse IgA anti-P-Cho (1:100; Sigma-Aldrich) or rabbit anti-serotype 4 (1:2,500; Statens Serum Institut) serum in PBS and incubated on ice for 30 min. Samples were quenched with 500 μl PBS and centrifuged at 4,000 rpm for 5 min. Pellets were resuspended in 100 μl of phycoerythrin-conjugated rat anti-mouse IgA (1:100) or FITC-conjugated goat anti-rabbit secondary antibodies in PBS and kept on ice in the dark for 30 min. Staining reactions were quenched with 500 μl PBS, and the mixtures were pelleted, resuspended

TABLE 1 Relevant strains and primers used in this study

Strain or primer	Description[a] or sequence (5′–3′)
Strains	
AC316 (TIGR4)	Wild-type serotype 4 strain, Gm^r
AC4421 (TIGR4Δ*cps*)	Serotype 4 strain with *cps* locus replaced with Cm^r cassette
MGS023 (TIGR4 AI45dup)	Serotype 4 strain with an in-frame addition of alanine and isoleucine at position 45 in Cps4E, Gm^r Sm^r
MGS024 (TIGR4 A42E)	Serotype 4 strain; mutation A42E in Cps4E, Gm^r Sm^r
MGS025 (TIGR4 A79E)	Serotype 4 strain; mutation A79E in Cps4E, Gm^r Sm^r
MGS026 (TIGR4 Y89*)	Serotype 4 strain; mutation Y89* in Cps4E, Gm^r Sm^r
MGS027 (TIGR4 D141*)	Serotype 4 strain; mutation D141* in Cps4E, Gm^r Sm^r
MGS028 (TIGR4 A156V)	Serotype 4 strain; mutation A156V in Cps4E, Gm^r Sm^r
MGS029 (TIGR4 G201S)	Serotype 4 strain; mutation G201S in Cps4E, Gm^r Sm^r
MGMV 3526	Serotype 19A, encapsulated conjunctival isolate
MGMV 3105	Serotype 19A, acapsular conjunctival isolate
MGS 3526 R377G	Serotype 19A, conjunctival isolate with R377G mutation in *cpsE*
MGS 3105 G377R	Serotype 19A, conjunctival isolate with G377R mutation in *cpsE*
Primers	
For amplifying and sequencing *cps4E*	
F *cps4D*	GCGATACTCGAAATTCAGTTATGTTA
R *cps4F*	CAAATATCTGACAACCTGAATGGTTCTGGC
F *cps4E* seq	CCTTTTCGTTTCAGTACGTAAGGT
For generating AI45dup PhaseLocked mutants	
F1 *cps4C*	CAACACTGGAGGAGGCAA
R1 PhaseLock2	AAGTTTAATCGCGATGGCAATTGCAATTATGATAAAAAC
F2 PhaseLock2	TTGCAATTGCCATCGCGATTAAACTTGATTCAGAAGGTCC
R2 *cps4F*	CCCAGGTATAGTACGAGAAACTATCGAG
R1 PhaseLock3	AAGTTTAATTGCGATGGCAATTGCAATTATGATAAAAAC
F2 PhaseLock3	TTGCAATTGCCATCGCAATTAAACTTGATTCAGAAGGTCC
For serotype 19A experiments	
F *cpsE*	ATGAGTGAAAAACTAGCAAAG
R *cpsE*	CTATTTCGCTCCATCCTTC
F *cpsE* seq	TCAAGTGGTATTGGAAGCGG
F1 *cpsB*	TATTCATTCGCACATCGTCT
R1 MGMV 3526 R377G	GTCCACTGTTGGTGGTCCTGTACCTACCAAACTCATATCTC
F2 MGMV 3526 R377G	TAGGTACAGGACCACCAACAGTGGACGAGTATGAGCACTATA
R1 MGMV 3105 G377R	GTCCACTGTTGGTGGTCCTGTACCTACCAAACTCATATCTC
F2 MGMV 3105 G377R	TAGGTACACGACCACCAACAGTGGACGAGTATGAGCACTATA
R2 *cpsH*	CACCCTCGTTTTCTTTCATCAA

[a] Gm^r, gentamicin resistant; Sm^r, streptomycin resistant; Cm^r, chloramphenicol resistant. * denotes a mutation that resulted in a stop codon.

in 300 μl 2% PFA, and analyzed as described above. All samples were collected (25,000 events) on a FACSCalibur analytical flow cytometer, analyzed, and plotted using Flowlogic (Inivai Technologies).

Epithelial adhesion and abiotic biofilm formation assays. For abiotic biofilm experiments, mid-exponential-phase cultures grown in THY were back-diluted to an optical density at 600 nm (OD_{600}) of 0.01 in fresh THY, and 200 μl was plated in 96-well plates in replicates of five and incubated for 18 h. Wells were washed three times with 250 μl PBS, stained with 250 μl of 0.05% (wt/vol) crystal violet in PBS, and incubated for 30 min at room temperature. Subsequently, the crystal violet solution was aspirated from the wells, and wells were washed once with 280 μl PBS. Stained biofilms were dissolved in 250 μl of 95% ethanol, and the OD was read at 570 nm.

For epithelial adhesion experiments, 1 ml of mid-exponential-phase cultures grown in THY was pelleted, washed in PBS, and resuspended to 10^7 CFU/ml in PBS. One milliliter of culture was added to triplicate wells in 24-well plates containing confluent monolayers of A549 human lung carcinoma cells, and plates were centrifuged for 5 min at 2,500 rpm to initiate contact between pneumococci and host cells and incubated at 37°C for 2 h. Nonadherent bacteria were aspirated off cells, and wells were washed thoroughly with 1 ml of PBS. Wells were then treated with 200 μl of 0.125% trypsin-EDTA and incubated for 5 min at 37°C. Next, 300 μl of 0.025% Triton X-100 was added to wells followed by thorough pipetting to lyse host cells. The released bacteria were serially diluted and plated on BA for enumeration. Data are presented as the percentage of input bacteria bound and are normalized to the calculated number of total input bacteria.

Animal infections. All experiments used female Swiss Webster mice, 6 to 9 weeks old (Taconic Laboratories). All animal experiments were performed in accordance with NIH guidelines, the Animal Welfare Act, and U.S. federal law. The experimental protocol used for this study was approved by the Tufts University School of Medicine's Institutional Animal Care and Use Committee. Mice used for these experiments were housed in an AAALAC-accredited research animal facility. Strains of interest were grown to mid-exponential phase in THY, pelleted for 4 min at 4,000 rpm, and washed in PBS. For blood infections, mice received ~10^3 or 10^8 CFU in 100 μl by intraperitoneal (i.p.) injection. Mice were euthanized 18 to 24 h postinfection by CO_2 asphyxiation. To recover bacteria from the blood,

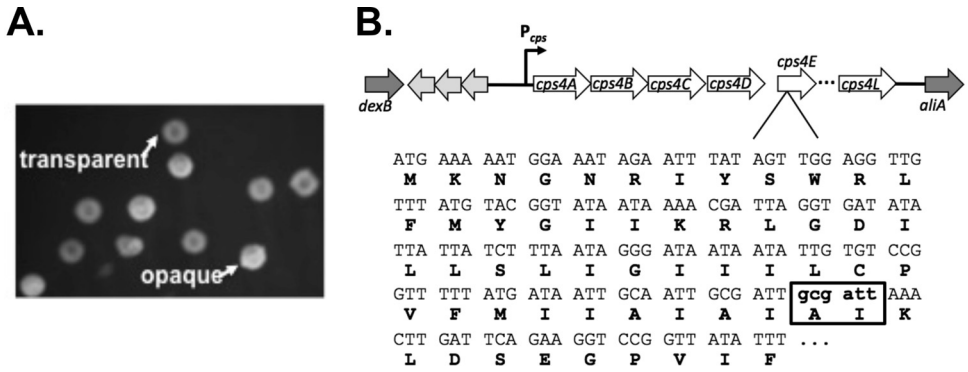

FIG 1 Observation of a subpopulation of cells with reduced capsule levels in a wild-type TIGR4 strain and its *cps4E* genotype. (A) Cells from the TIGR4 sample with a heterogeneous capsule phenotype were plated on TSA supplemented with catalase and were incubated overnight at 37°C with CO_2. Colony morphology was examined using oblique transmitted light microscopy. Arrows denote distinct transparent and opaque colonies. (B) Genomic DNA was isolated from bacteria exhibiting transparent or opaque phenotypes, and the *cps4E* gene was sequenced and aligned to a TIGR4 reference genome. The schematic shows the TIGR4 *cps* locus and location of the 6-bp, in-frame insertion of the sequence GCGATT present in transparent bacteria. The arrowhead upstream of *cps4A* represents the *cps* promoter, P_{cps}. The light gray arrows are three IS elements upstream of the *cps* locus.

500 μl of blood was removed by cardiac puncture, and clotting was prevented by the addition of 3 μl of 500 mM EDTA. Serial dilutions of recovered bacteria from each mouse were plated on BA, supplemented with Sm (100 μg/ml) when appropriate.

India ink staining for visualizing capsule. To qualitatively visualize the pneumococcal capsule in strains of interest, 300 μl of mid-exponential-phase bacteria grown in THY were pelleted and washed with PBS, and pellets were resuspended in 100 μl of PBS. India ink wet mounts were made by adding 8 μl of bacteria to 2 μl of India ink and were visualized on a Nikon Eclipse 80i with a 100× objective. Images were captured using a Photometrics CoolSnap HQ camera, and pictures were visualized using Roper Scientific software. When this negative-staining technique is used, the pneumococcal cell appears dark and is surrounded by a white halo, which is a result of the exclusion of India ink particles by the capsule.

Statistical analysis. Wilcoxon-signed rank tests and one-way analysis of variance (ANOVA) were performed where indicated using GraphPad Prism (GraphPad Software, Inc.).

RESULTS

Analysis of a spontaneous TIGR4 mutant with reduced capsular polysaccharide. During a routine measurement of capsule expression in a culture of wild-type (WT) TIGR4 by flow cytometry, we noticed there was an unusual subpopulation of cells exhibiting reduced capsule level (data not shown). When we plated a portion of this culture on TSA and examined colonies by oblique transmitted light microscopy, we observed a heterogeneous population with distinct transparent and opaque colony morphologies (Fig. 1A). The two colony types were genetically stable, yielding high and low capsule expression, respectively, for the opaque and transparent types (data not shown). Since mutations in the *cpsE* gene, which encodes the initiating glycosyltransferase (38), were reported to result in a reduction of capsule expression (39, 41, 42), we sequenced this gene in both transparent and opaque variants. These data revealed that, while the opaque strain had a wild-type *cps4E* sequence, the transparent variant possessed a duplication of a 6-nucleotide sequence, GCGATT, which results in the addition of an alanine and isoleucine after amino acid 45 (Fig. 1B; also, see Fig. 4B). We refer to this strain as the AI45dup-containing mutant. Interestingly, in a phase-variable serotype 8 strain of pneumococcus, caused by a 223-bp sequence duplication event in *cpsE* that yields a premature stop codon and acapsular phenotype, the duplication sequence also starts with the GCGATT sequence (41), which led us to speculate that *cps4E* was also a phase-variable locus. Our subsequent *in vitro* and *in vivo* experiments aimed to elucidate the functional consequences of reduced encapsulation in the AI45dup-containing mutant strain.

The AI45dup mutation does not result in complete loss of capsule. However, by flow-cytometric analysis, this strain possesses an approximately 10-fold reduction in capsule compared to the WT (Fig. 2A). Since an inverse correlation between exposed P-Cho and capsule expression was reported (47), we stained AI45dup-containing mutant cells with a P-Cho-specific monoclonal antibody (TEPC-15) to determine its P-Cho phenotype. Consistent with having a reduced capsule level, the AI45dup-containing strain exhibits more exposed P-Cho than the WT strain and has a phenotype more similar to that of the TIGR4Δ*cps* strain (Fig. 2B). In order to examine the functional consequences of the AI45dup mutation, we tested this strain in abiotic, static biofilm and epithelial cell adhesion assays. Since a reduction in capsular polysaccharide results in the exposure of underlying surface molecules involved in biofilm formation (13) and epithelial cell adhesion (48–50), we anticipated that the AI45dup-containing mutant would demonstrate an enhanced ability to form abiotic biofilms and bind host cells. As shown in Fig. 2C and D, the AI45dup-containing mutant is significantly better than WT TIGR4 at forming abiotic biofilms and adhering to A549 human lung carcinoma cells. While the AI45dup-containing strain was comparably better than even the TIGR4Δ*cps* strain, this difference was not statistically different in either assay.

Capsule level has also been reported to influence susceptibility to complement deposition, opsonophagocytosis, and virulence in models of sepsis. Less encapsulated strains are more susceptible to C3 deposition and neutrophil-mediated killing, while more encapsulated strains are more resistant to these processes and are also more virulent during blood infections (7, 9, 51). Consistent with these prior reports, as determined by flow cytometry, the mean fluorescence intensity (MFI) for $C3^+$ bacterial cells from the AI45dup-containing mutant is approximately 200-fold higher than that of WT cells, indicating that this mutant is drastically more susceptible to C3 deposition (Fig. 2E). Since capsule is indispensable for causing disease in the blood (2, 52), we tested how the reduced level of capsular polysaccharide in the AI45dup-con-

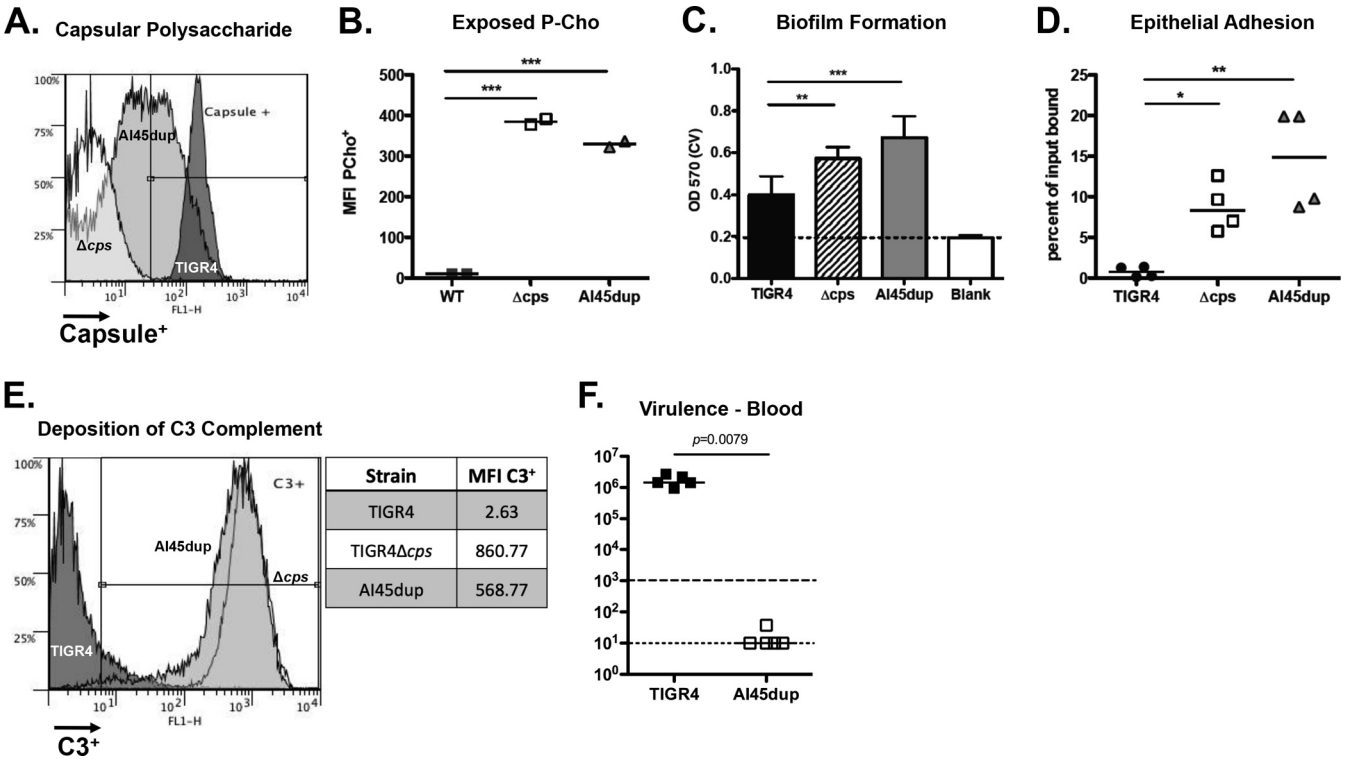

FIG 2 *In vitro* and *in vivo* characterization of the AI45dup *cps4E* mutant. For all experiments described below, bacteria were grown to mid-exponential phase in THY-Oxyrase and then subjected to the appropriate assay. WT TIGR4, TIGR4Δ*cps*, and AI45dup bacteria were stained with anti-capsular polysaccharide (serotype 4) (A) or anti-P-Cho (TEPC-15) (B) antibodies and subjected to FACS analysis. Data for capsule levels are representative histogram plots from 4 independent experiments with similar results. Data for exposed P-Cho are from two independent experiments; the horizontal lines are the means. Data are presented as mean fluorescence intensity (MFI). (C) For abiotic, static biofilm experiments, bacteria were washed in 1× PBS, diluted to an OD_{600} of 0.01, plated in 96-well plates, and incubated at 37°C with CO_2 for 18 h. Data are from one experiment representative of 3 independent experiments and are means and standard deviations (SD) from five technical replicates. (D) In epithelial adhesion experiments, bacteria were added to a monolayer of A549 human lung carcinoma epithelial cells at a multiplicity of infection (MOI) of 50:1 and incubated for 2 h at 37°C. Adherent bacteria were recovered and enumerated on blood agar plates, and results are presented as percent bound normalized to the number of input bacteria. Data are from 4 independent experiments, with the horizontal bar denoting the mean. (E) For C3 deposition experiments, bacteria were opsonized with infant rabbit serum for 30 min at 37°C with rolling, stained with a FITC-conjugated anti-C3 complement antibody, and analyzed by flow cytometry. Data are representative histograms from 4 independent experiments. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared to TIGR4 using one-way ANOVA. (F) Virulence of WT TIGR4 and AI45dup-containing strains was tested in a blood model of infection. For each strain, 10^3 CFU were injected i.p. into Swiss Webster mice. After 18 h, mice were euthanized, and bacterial counts in blood were determined by plating for CFU. The horizontal bar shows the median for each group and each point represents an individual mouse. Open symbols indicate a mouse that yielded no detectable CFU, and the lower dotted line represents the limit of detection. The exact *P* value was calculated using Student's *t* test.

taining mutant would affect its virulence in a mouse model of sepsis. Mice were infected with either WT TIGR4 or the mutant strain, and bacterial burden in the blood was determined after 18 h. Strikingly, the AI45dup-containing mutant was completely avirulent at an infectious dose sufficient to cause significant morbidity using the WT strain (Fig. 2F).

Phenotypic and genetic reversion of the AI45dup-containing mutant to wild type following blood infection. The duplication of GCGATT in *cps4E* results in the formation of a short sequence repeat (DR2) (Fig. 3A), which we predicted might be unstable in a manner akin to phase-variable loss of a repeat through slipped-strand mispairing (53–55). Note that another direct repeat (DR1) is present upstream and overlaps DR2 (Fig. 3A). The loss of one repeat (either DR1 or DR2) would be expected to restore Cps4E to the wild-type sequence and thus wild-type capsule expression and virulence during sepsis. Therefore, we tested the AI45dup-containing mutant strain in this *in vivo* niche to determine if, under this selective pressure, it is capable of reverting back to its fully encapsulated state. For these experiments, we colony purified four independent AI45dup clones and injected each i.p. into two Swiss Webster mice at 10^8 CFU per mouse. After 24 h, mice were euthanized, bacteria were recovered from the blood and plated on TSA, and colony morphologies were examined using oblique transmitted light microscopy. Each mouse output yielded a mixture of transparent and opaque colonies. For each mouse, two colonies were isolated and restreaked to confirm colony phenotype, and DNA was prepared for sequencing of the *cps4E* gene. A total of 26 colonies from 13 mice in two independent experiments were analyzed in this portion of the study.

The data from these experiments revealed that all 16 morphologically opaque colonies had the Cps4E amino acid sequence restored to the wild type, while transparent colonies retained the parental AI45dup sequence. Interestingly, among opaque colonies with the restored Cps4E sequence, there were two distinct *cps4E* genotypes, either precise deletion of one DR1 repeat (revertant genotype 1) or precise deletion of one DR2 repeat (revertant genotype 2) (Fig. 3A). In epithelial cell adhesion assays, we determined that the opaque revertants exhibited the expected poor

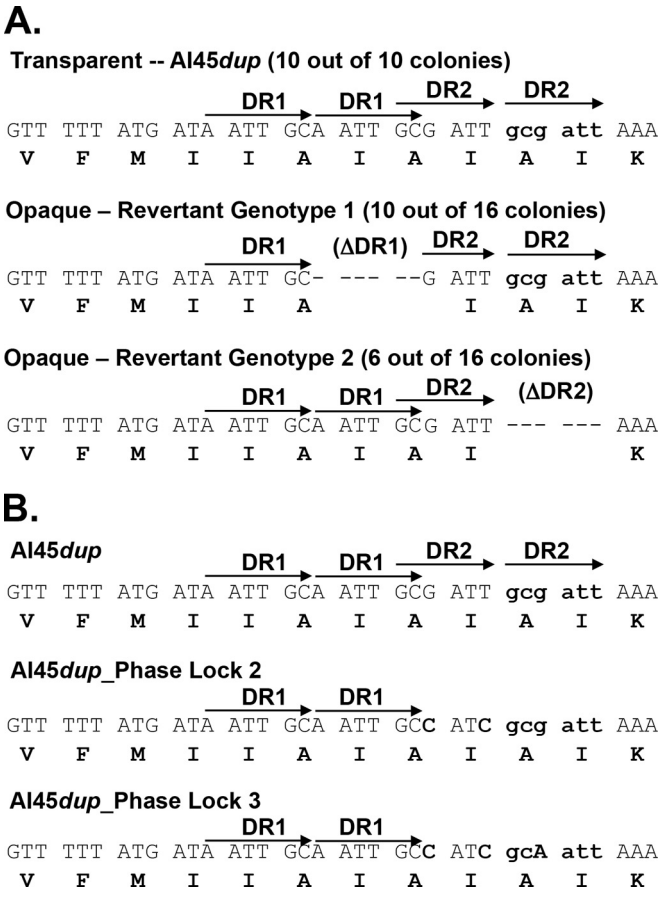

FIG 3 Evaluating the reversibility of the AI45dup-containing mutant following blood infection. (A) Genotype and corresponding translation of a portion of the *cps4E* gene in the AI45dup-containing strain. The 6-bp sequence duplication is in lowercase. After *in vivo* blood infection, recovered output bacteria were isolated and DNA was isolated from colonies with opaque and transparent morphology. The *cps4E* gene was sequenced and aligned with that from the WT TIGR4 reference strain. All 16 of the analyzed opaque revertants that recovered capsule had one of two different *cps4E* genotypes, precise excision of either the 6-bp duplication (ΔDR2; 6 of 16 colonies) or of the upstream sequence, AATTGC (ΔDR1; 10 of 16 colonies). All 10 of the analyzed transparent colonies retained the second DR2 repeat. (B) Genotype and corresponding translation of a portion of *cps4E* in the AI45dup, AI45dup PhaseLock 2 (AI45dupPL2), and AI45dupPL3 strains. Following i.p. blood infection with either AI45dupPL2 or AI45dupPL3 strains, all recovered output bacteria failed to revert and retained their phase-locked genotype and transparent phenotype.

A.

Colony Phenotype	Number of Colonies Sequenced	Number of colonies with mutations in *cps4e*
Unpassaged	10	0
Passaged - Opaque	33	0
Passaged - Transparent	26	22
Number of Mutants	**Nature of Mutation**	**Result**
1	2nt deletion	Frameshift (Stop)
2	SNP	Nonsense
19	SNP	Missense

B.

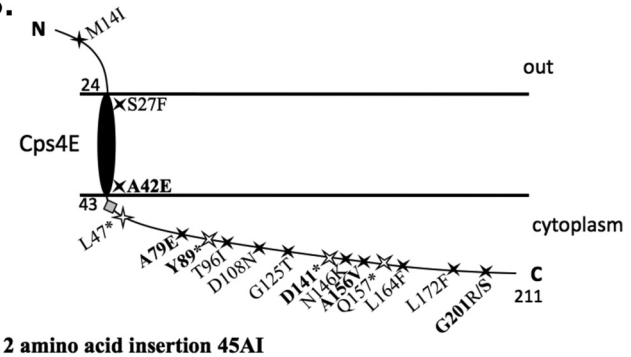

FIG 4 *cps4E* genotypes of the resulting reduced capsule mutants isolated from *in vitro* passaging. (A) Twenty-six transparent colonies were identified and colony purified onto new TSA plates to confirm their colony morphology. As controls, unpassaged and opaque colonies were also studied. Genomic DNA was isolated from these strains, and forward and reverse primers flanking *cps4E* were used for PCR. (A) Sequencing results. (Top) Colony phenotype, number of isolates of each colony phenotype sequenced, and the number of colonies with mutations in *cps4E*. (Bottom) For the transparent colonies with mutations in *cps4E*, the nature of the mutation and the phenotype conferred are provided. (B) The topology of Cps4E was determined using the TMpred program from the ExPASy Proteomics website (http://www.expasy.org/tools). Depicted are the locations of the mutations, including the originally described 6-bp insertion. Mutants in bold were further characterized in subsequent experiments.

binding phenotype identical to that of the WT TIGR4 strain (data not shown).

We speculated that slipped-strand mispairing of the DNA polymerase along the DR1 short sequence repeat may have led to the initial spontaneous duplication event that gave rise to the AI45dup-containing mutant strain. However, our failed attempt to reproduce this mutant (described below) suggested that it is a rare event. In contrast, under selective pressure in blood, the AI45dup allele readily gave rise to reversion events involving deletion of one or the other repeat. We tested whether slipped-strand mispairing was the mechanism responsible for reversion of the *cps4E* gene by generating two "phase-locked" (PL) mutants in the AI45dup background. These mutants had two (AI45dupPL2) or three (AI45dupPL3) mutated nucleotides in the second DR2 repeat, while the amino acid sequence remained unchanged, and care was taken not to introduce rare codons (Fig. 3B).

For these experiments, two colony-purified clones for each of the AI45dupPL2 and AI45dupPL3 strains were inoculated into two or three mice each via i.p. injection as described above. Subsequently, in order to examine colony morphology, bacteria were recovered from the blood and plated on TSA. All of the output colonies examined, 21 colonies for AI45dupPL2 and 16 colonies for AI45dupPL3, demonstrated a transparent colony phenotype, and each retained the parental *cps4E* sequence. These data suggest that the DR2 repeat is required for effective phenotypic and genetic reversion of the AI45dup-containing mutant. Because phase-locking of DR2 abrogated both types of deletion events, we did not test phase-locked mutations in DR1, though presumably this repeat is necessary in combination with the DR2 repeat for deletion of one DR1 repeat (revertant genotype 1) via slipped-strand mispairing.

Serial passaging of TIGR4 and characterization of generated capsule mutants. To determine if the duplication of GCGATT in *cps4E* was a frequent mutational event, we serially passaged 96 clones of WT TIGR4 *in vitro* in THY and on blood agar plates a total of eight times. We hypothesized that growth under these

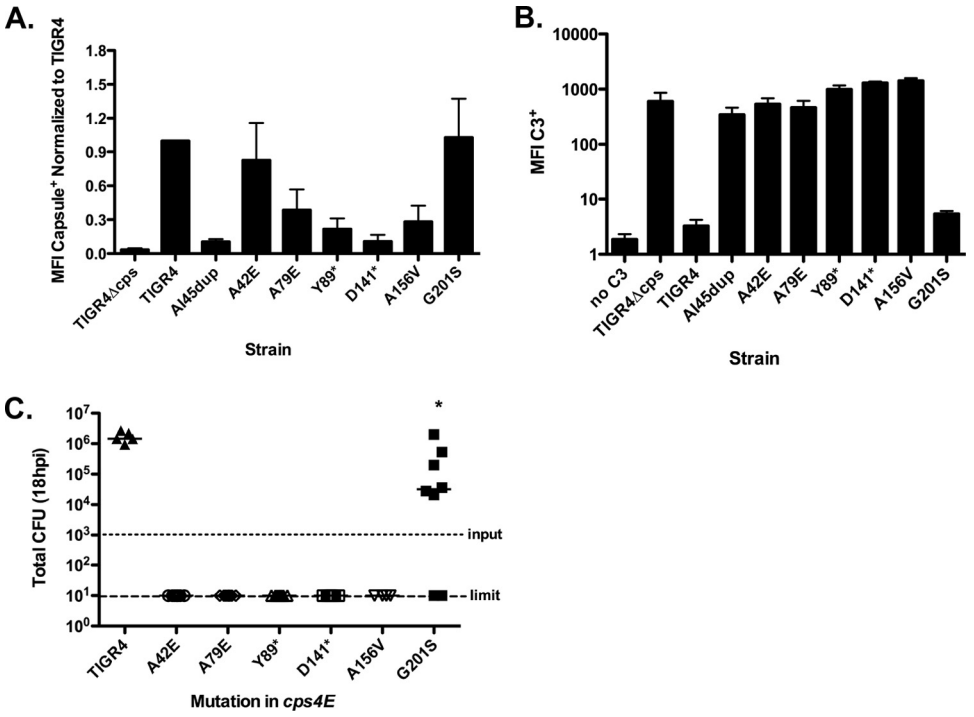

FIG 5 Characterization of a panel of *cps4E* capsule mutants obtained from serial passaging. Six representative *cps4E* mutants were selected for further *in vitro* and *in vivo* characterization. Mid-exponential-phase bacteria were subjected to FACS analysis of capsule levels (A) and C3 complement deposition (B). For capsule levels and C3 deposition, data are means and standard errors of the means (SEM) from three independent experiments. Capsule levels are presented as relative units normalized to levels in WT TIGR4. (C) Each of the 6 *cps4E* mutants was tested for virulence in a blood model of infection. Mid-exponential-phase bacteria were prepared and washed in 1× PBS, and ~10^3 CFU were injected i.p. into Swiss Webster mice. After 18 h, mice were euthanized, and blood was isolated via cardiac puncture and plated on BA plated for enumeration of recovered bacteria. For all tested strains, except for the G201S strain, we failed to recover bacteria after infection. The G201S strain was capable of infecting animals, but we recovered significantly fewer bacteria than with WT TIGR4. Each point represents an individual animal, and the horizontal line denotes the median. The input number of bacteria and limit of detection for this experiment are indicated by the dotted lines. Data were pooled from 2 independent experiments. *, $P < 0.05$.

conditions would select for less encapsulated variants, since acapsular strains of pneumococcus exhibit a growth advantage *in vitro* (40, 56). After serial passage, 26 of the 96 clones exhibited transparent colony morphologies (Fig. 4A). In order to examine the molecular basis of this phenotype, DNA was isolated from these clones, along with 10 opaque control (unpassaged) and 33 opaque (passaged) colonies, and *cps4E* was sequenced. Strikingly, 22 out of 26 transparent colonies harbored mutations in *cps4E*, with 16 of these mutations being unique (Fig. 4). However, none of these mutants contained the originally described GCGATT sequence duplication. Instead, the majority of the mutants contained single missense mutations, while two mutants harbored a nonsense mutation, and a single mutant possessed a 2-bp deletion resulting in a frameshift and early stop codon (Fig. 4A and B). Based on the predicted protein topology generated by the program TMpred, the majority of the mutations were located in the cytoplasmic portion of Cps4E. Two mutations mapped to the transmembrane region, and a single mutation was present in the predicted extracellular part of the protein (Fig. 4B). From these results, we conclude that the AI45dup transparent mutant arises from a rare duplication event but, once formed, is readily revertible back to an opaque colony type when subjected to strong selection in an animal host.

Six representative mutants (Fig. 4B, boldface) were characterized with respect to capsule expression level and associated phenotypes. Flow-cytometric analysis revealed that four of the six mutants had reduced capsule levels, while two mutants, the A42E and G201S mutants, exhibited seemingly WT levels of capsule, though there was a fair amount of variability observed in these two mutants (Fig. 5A). When the mutants were tested for C3 complement deposition, all except the G201S mutant exhibited higher C3 deposition, consistent with their having reduced capsule expression (Fig. 5B). Finally, when they were tested in the murine model of sepsis, which is the most stringent of the three assays for capsule expression level, we observed that all six mutants were attenuated (Fig. 5C). The G201S mutant was only mildly attenuated, consistent with its near-wild-type capsule expression level.

To determine if the more frequent SNP class of mutations in *cps4E* were also revertible, each mutant was subjected to the identical *in vivo* passaging scheme in the blood as detailed above for the AI45dup-containing mutant. We predicted that the spontaneous SNP mutations in *cps4E* would be less capable of doing so due to the lack of short sequence repeats associated with them that could revert by slipped-strand mispairing. Interestingly, despite maintaining the SNP in *cps4E* (Table 2), the G201S strain was lethal at the high dose used for this experiment, which may be due to its near-wild-type level of capsule (Fig. 5A). Consistent with our hypothesis, of the remaining SNP mutants, none could revert, with the one exception of the Y89* strain (Table 2). All of the other tested SNP mutants were either completely avirulent (no recovered bacteria) or yielded low numbers of recovered bacteria, all of which were transparent and maintained the mutation in *cps4E*

TABLE 2 Genotypes and colony phenotypes of parental *cps4E* mutants and output bacteria after blood infection

Mutation in Cps4E	Colony phenotype of parental strain	*cps4E* genotype after blood infection	Colony phenotype after blood infection
A42E	Transparent	Maintains SNP	Transparent
A79E	Transparent	Maintains SNP	Transparent
Y89*	Transparent	Reverts to WT allele	Opaque
D141*	Transparent	Avirulent or maintains SNP	Avirulent/maintains SNP; transparent
A156V	Transparent	Avirulent	NA[a]
G201S	Transparent	Maintains SNP; reduced virulence	Transparent

[a] NA, not applicable. Since the A156V mutant strain was completely avirulent in the blood, it was not possible to determine the colony phenotype of recovered bacteria.

(Table 2). Reversion of the Y89* strain was variable, occurring in only half the animals. It is unclear to us why this SNP mutant was capable of reverting.

Mutations in *cpsE* in a conjunctival isolate. Most pneumococcal conjunctival infections are caused by acapsular or nontypeable strains that lack most of the *cps* operon, which are referred to as group I NT strains. However, in relatively rare group II NT strains, the *cps* locus remains largely intact, though the strains are acapsular (16-18, 21, 23, 57-60). Based on our observations above, we hypothesized that the molecular mechanism responsible for loss of capsule in group II NT conjunctival isolates was due to a mutation(s) in *cpsE*. We tested this hypothesis using available isolates lacking *cps* operon deletions; two serotype 19A conjunctival isolates, one that was encapsulated (3526) and another that lacked capsule (3105) (Fig. 6A, top). Upon comparing the sequence of *cpsE* between these two strains, we discovered four missense mutations in the acapsular strain. To determine if these mutations were responsible for the acapsular phenotype, we swapped the *cpsE* alleles between the two conjunctival strains. These experiments revealed that this was sufficient to switch the capsular phenotypes (data not shown). Upon further examination, we determined that the missense mutation at base 1129, which changed an arginine to a glycine at position 377 in the protein, was necessary and sufficient for the acapsular phenotype. Of note, in the majority of pneumococcal serotypes, including 19A, the CpsE protein is 455 amino acids, while in TIGR4 (serotype 4), it is a much smaller, 211-amino-acid protein. Specifically, by making the G377R mutation in *cpsE* (yielding the 3105 G377R strain), we were able to confer capsule upon the 3105 acapsular isolate. Conversely, the 3526 R377G mutation rendered the encapsulated isolate unencapsulated (Fig. 6A, bottom). Notably, this is the same critical amino acid residue in CpsE identified in a serotype 18C acapsular nasopharyngeal isolate (40). Finally, we tested whether restoring capsule to the acapsular conjunctival isolate was sufficient to make the strain virulent in the blood. Consistent with capsule expression level, the unencapsulated conjunctival isolate 3105 was completely avirulent at a dose of 10^8 CFU/mouse, while the 3105 G377R encapsulated isogenic derivative was lethal in mice at the same dose (Fig. 6B).

DISCUSSION

Several previous studies demonstrated that pneumococcus needs to modulate levels of capsular polysaccharide in order to successfully colonize different host niches, including the nasopharynx, lung, blood, and conjunctiva (1, 7, 16, 61). However, it is still incompletely understood how this is accomplished: a phosphorelay system comprised of CpsB, CpsC, and CpsD and transcriptional regulation of capsule genes comprise two levels of control of capsule levels (33–35, 62, 63). This study and work by others supports the notion that there exists another potential layer of control, involving mutations in the gene encoding the initiating glycosyltransferase, *cpsE* (39, 40). Our experiments using serotype-matched acapsular and encapsulated conjunctival isolates support the idea that this level of control occurs in nature.

Our initial identification of a less encapsulated TIGR4 mutant caused by a spontaneous 6-bp duplication in *cps4E* led to the hypothesis that this phenotype arose due to slipped-strand mispairing along a short sequence repeat immediately upstream. This form of genome plasticity was reported for other genes in pneumococcus (55), as well as in other mucosal pathogens, including *Neisseria* species, *Streptococcus agalactiae*, *Haemophilus influenzae*, and *Helicobacter pylori* (53, 54, 64–67). This notion was further supported by similar observations made in three other pneumococcal serotypes (types 3, 37, and 8), which also arose due to spontaneous tandem sequence duplications in capsule biosynthesis genes, including a 223-bp duplication in *cps8E* (41, 42). We suspect that mutations in *cpsE* occur at a higher frequency, since mutations in other serotype-specific genes are toxic, presumably due to the accumulation of intracellular capsule precursors, which are alleviated by a suppressor mutation in *cpsE* (68). In contrast to

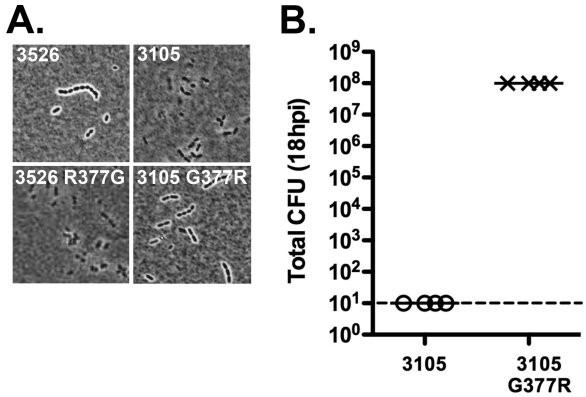

FIG 6 The acapsular conjunctival isolate contains a mutation in *cpsE*, and a residue at position 377 controls the phenotype. (A) (Top) Encapsulated (3526) and acapsular (3105) serotype 19A conjunctival isolates were grown to mid-exponential phase in THY-Oxyrase, stained with India ink, and visualized by microscopy to verify their capsule status. Four missense mutations in *cpsE* in strain 3105 were identified when its sequence was compared to that of strain 3526. Of particular importance are arginine 377 (strain 3526, encapsulated) and glycine 377 (strain 3105, acapsular). (Bottom) Swapping a single amino acid residue at position 377 is sufficient for controlling the capsular phenotype; 3526 R377G becomes acapsular, while 3105 G377R regains capsule. (B) The virulence of strain 3105 (acapsular) and 3105 G377R (encapsulated) was tested in a blood model of infection. For these experiments, each strain was grown to mid-exponential phase in THY-Oxyrase, washed in 1× PBS, and 10^8 CFU were injected i.p. into Swiss Webster mice. After 18 h mice, were euthanized, and blood was collected via cardiac puncture and plated on BA for enumeration of bacteria. While strain 3105 (acapsular) was completely avirulent, its encapsulated counterpart, 3105 G377R, was capable of causing disease at this infectious dose. Each point represents an individual mouse. Open symbols indicate mice that yielded no detectable CFU, and the dashed line represents the limit of detection.

a complete loss of capsule reported for these strains, the AI45dup-containing mutant retained a low level of capsular polysaccharide. This suggests that this two-amino-acid-duplication mutant retains some glycosyltransferase activity. A thorough analysis of *cpsE* in D39 (serotype 2) identified several residues in the extracytoplasmic loop (amino acids 117 to 264) and cytoplasmic tail (amino acids 284 to 455) that were critical for glycosyltransferase activity (39). In D39 and most other serogroups, including 19A, CpsE is a large protein, approximately 455 amino acids, while TIGR4 is unique in coding for a smaller CpsE protein, of 211 amino acids. However, the TIGR4 CpsE is highly homologous (33% identity) across its entire length to the C-terminal half of these longer CpsE proteins, which includes their final transmembrane portion and cytoplasmic tail, believed to have a glycosyltransferase function. Consistent with this, spontaneous null mutations in the TIGR4, D39, and 19A *cpsE* genes all cluster predominately in the portion corresponding to this C-terminal cytoplasmic tail (39; also this study).

Pneumococcal strains with reduced levels of capsular polysaccharide were previously shown to more effectively form biofilm structures and adhere to host epithelial cells (10, 15, 69). In support of these findings, the AI45dup-containing strain exhibited an enhanced ability to form static biofilms *in vitro* and adhere to a human epithelial cell line. Additionally, a reduction in capsule level was found to correlate with more exposed P-Cho and increased susceptibility to C3 complement deposition (7, 48, 51), and we showed that this is true as well for the AI45dup-containing strain. The AI45dup-containing mutant was completely avirulent in a mouse model of sepsis. In addition to C3 complement-mediated opsonophagocytic killing, high concentrations of C-reactive protein (CRP) found in the blood can bind exposed P-Cho residues present in the AI45dup-containing strain and initiate the classical complement cascade, likely resulting in complete clearance of the mutant (51, 70).

Phase variation is a heritable and reversible genetic event that occurs at a relatively high frequency, which results in phenotypic heterogeneity within a population (71, 72). Slippage of the DNA polymerase along short sequence repeats, referred to as slipped-strand mispairing, is a major mechanism of phase variation in bacteria. The AI45dup mutation fit the phase variable criterion of being reversible, since after one passage in the bloodstream of mice, we readily isolated two types of repeat sequence deletion mutants that restored Cps4E to the wild-type amino acid sequence with concomitant restoration of capsule expression. Furthermore, by generating phase-locked mutants and showing that these were incapable of reverting, we hypothesize that reversion of the AI45dup-containing mutant occurs by a slipped-strain mispairing mechanism.

Despite our findings that the AI45dup mutation fulfilled the requirement of being reversible, it was determined that the duplication event that gave rise to this mutant in the first place is rare, being undetectable among a large panel of spontaneous mutants selected for having reduced capsule expression. Thus, we conclude that the AI45dup mutation is not a true phase-variable allele in pneumococcus. Nevertheless, in our attempt to reisolate the AI45dup mutation through selection, we identified several strains with unique SNPs in *cps4E* resulting in missense and nonsense mutations that reduced or in some cases abrogated capsule expression. Analysis of a subset of these mutants revealed that levels of capsular polysaccharide and extent of C3 complement deposition could serve as a predictor of virulence in the blood. These findings align with those of other studies reporting that capsule serves as a critical barrier that impedes C3 deposition and that more encapsulated strains (e.g., opaque variants) are more invasive and/or pathogenic in the blood (7, 9, 73). Additional studies revealed that in contrast to the AI45dup-containing mutant, with the exception of the Y89* strain, the vast majority of the *cps4E* point mutants were incapable of reverting or suppressing their mutation to restore capsule level and virulence. In half of the animals (4 out of 8) infected with the Y89* strain, we recovered bacteria with the wild-type *cps4E* allele. Since there were no obvious DNA sequences surrounding this mutation that would be susceptible to slipped-strand mispairing, it is difficult to speculate why this particular SNP mutant could revert. Overall, our results indicate that the spontaneous mutations in *cpsE* observed in this study are not phase variable but instead may serve as a form of adaptive mutation by reducing or eliminating capsule production.

In order to determine if our studies were applicable to clinically relevant strains, we obtained two serotype 19A conjunctival isolates, since conjunctivitis is often caused by acapsular pneumococcal strains (16, 18). Indeed, we found that a single missense mutation in the *cpsE* gene was responsible for the acapsular phenotype. It would be informative to evaluate additional conjunctival isolates for mutations in *cpsE*. However, the fact that the majority of acapsular conjunctival isolates completely lack the *cps* operon (21-23, 74) makes a study of this nature difficult to undertake.

In conclusion, this study revealed that mutations in *cpsE*, which encodes the initiating glycosyltransferase, ranging from spontaneous sequence duplication to nonsense and missense mutations, were capable of reducing capsule level to various degrees. We propose that these mutations are adaptive in that they provide an additional layer of adjusting capsular polysaccharide expression to allow optimal pneumococcal colonization in niches such as the conjunctiva, where capsule is a detriment.

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